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MicroRNA modulation of megakaryoblast fate involves cholinergic signaling

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Abstract

MicroRNAs (miRNAs) are abundant small regulatory RNAs with multiple roles in cell fate determination. The processes regulating cellular miRNA levels are still unclear and experimental oligonucleotide tools to readily mimic their effects are not yet available. Here, we report that thapsigargin-induced intracellular Ca^{++} release suppressed pre-miR-181a levels in human promegakaryotic Meg-01 cells, induced differentiation-associated nuclear endoreduplication and caspase-3 activation and replaced the acetylcholinesterase 3′ splice variant AChE-S with AChE-R. AChE, PKC and PKA inhibitors all attenuated the pre-miR-181a decline and the induced differentiation. AChmiON, a synthetic 23-mer 2′-oxymethylated oligonucleotide mimicking the miR-181a sequence, blocked the calcium-induced differentiation while elevating cellular pre-miR-181a levels and inducing DNA fragmentation and cell death. Moreover, when added to RW 264.7 macrophages, AChmiON at 100 nM induced nitric oxide production with efficiency close to that of bacterial endotoxin, demonstrating physiologically relevant activities also in blood-born monocytes/macrophages. The stress-induced modulation of hematopoietic miR-181a levels through AChE, PKC and PKA cascade(s) suggests using miRNA mimics for diverting the fate of hematopoietic tumor cells towards differentiation and/or apoptosis.

Keywords: MicroRNA; miR-181; Megakaryocytopoiesis; Macrophage; Nitric oxide; Differentiation; Hematopoiesis; Acetylcholinesterase; Calcium; Apoptosis; Oligonucleotide; Caspase

1. Introduction

MicroRNAs (miRNAs), 20–28 nucleotide (nt) non-coding RNAs regulate gene expression and cellular fate determination in multiple organisms [1–3]. The primary miRNA transcripts are processed by the nuclear RNase III Drosha, and the resulting ∼60–120 nt hairpin precursors are exported from the nucleus via the exportin-5 mechanism. In the cytoplasm, these precursors are further cleaved by Dicer, a double-stranded RNA endoribonuclease, to yield the mature miRNAs. These are incorporated into the RNA-induced silencing complex (RISC) for subsequent targeting to mRNAs, repressing their levels and/or suppressing translation (reviewed by Refs. [4,5]).

MiRNA functions in humans include regulatory roles in cell proliferation and hematopoiesis. Thus, *miR*-15 and *miR*-16 are often lost in chronic lymphocytic leukemia (CLL) [6]; *miR*-143 and *miR*-145 are consistently reduced in colorectal neoplasia [7]; the *miR*-142, *miR*-15 and *miR*-16 genes map to translocation breakpoints or deletions linked to human leukemias [6]; *miR*-181 was shown to increase B-lineage cells differentiation when expressed in hematopoietic stem/progenitor cells [2] but was downregulated under TPA-induced differentiation of HL-60 human promyelocytic leukemia cells to monocyte/macrophage-like cells [8]. Also, antisense suppression of miR-181a, a member of the miR-181 family, induced cell proliferation of the lung carcinoma cell line, A549 [9]. However, neither the cellular signals inducing changes in the levels of this and other miRNAs, nor the molecular processes promoted by such changes are yet understood.

Differentiation of cells implies a progressive restriction of the developmental potential, expression of a specific

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proteome through transcriptional and post-transcriptional changes and increasing specialization of function. In particular, hematopoietic differentiation of multipotential stem cells into the different blood cell lineages is controlled by the interplay of regulatory pathways that together determine the dynamic balance between proliferation, commitment and death. Deciphering the involvement of specific miRNAs in these pathways can elucidate their cellular function and yield novel RNA-targeted means to control these events. With these aims in mind, we selected the human Meg-01 promegakaryocytic tumor cells capable of differentiating into megakaryocyte-like cells (MK) [10]. MKs, the direct platelet precursors, undergo a nuclear endoreduplication process without cytokinesis [11], then complete their cytoplasmic maturation by undergoing profound cytoskeletal changes [12], form cytoplasmic extensions termed proplatelets and finally shed platelets in a process involving activation of caspase-3 and caspase-9 [13].

Like many other hematopoietic cell types, the differentiation of Meg-01 cells is subject to both stress and cholinergic signals [14]. Accordingly, Meg-01 cells express several splice variants of the acetylcholine hydrolyzing enzyme acetylcholinesterase (AChE), also known to exert non-catalytic effects in several processes, such as cell death and hematopoiesis [15,16]. Particularly, the "synaptic" (S) and the "read-through" (R) variants differ in their cellular localization, organization into multimers and non-catalytic roles in neurons and blood cells [17]. In murine erythroleukemia (MEL) cells, AChE-R mRNA levels increase under dimethyl sulfoxide-induced differentiation [18]. Thus, like miR-181 family members, AChE-R levels are involved in cell fate decisions.

MiR-181a was recently shown to be expressed in brain neurons [19], actually designated as *miR*-178 in this particular work) where AChE-R mRNA levels increase under psychological stress responses involving intracellular Ca^{++} release [20–22]. Therefore, we explored the possibility that enforced intracellular release of Ca^{++} stores in Meg-01 cells could affect their miR-181a levels. To emulate the activities of miR-181a, we developed a synthetic oligonucleotide mimic of miR-181a termed AChmiON and tested its involvement in Meg-01 differentiation, DNA synthesis, AChE gene expression and survival. Our findings demonstrate cholinergic regulation of miR-181a expression in Meg-01 cells and present miRNA mimics as a prospective RNA-based tools for leukemia research and therapeutic strategy.

2. Materials and methods

Materials: The hematopoietically active human AChE-R C-terminal peptide ARP (1-GMQGPAGSGWEEGSGSPPG-VTPLFSP-26) was from the American Peptide Company (Sunnyvale, CA, USA). The AChE-S C-terminal peptide ASP (1-DTLDEAERQWKAEFHRWSSYMVHWKNQFD-HYSKQDRCSDL-40) was prepared as detailed elsewhere [23]. Bisindolylmaleimide (BIM), *N*-(2-((*p*-bromocinnamyl) amino) ethyl)-5-isoquinolinesulfonamide (H89) and 3-carboxymethyl-17-methoxy-6,18,21-trimethyldocosa-2,4,8,12, 18,20-heptaenedioic acid (bongkrekic acid) were from Calbiochem (San Diego, California, USA). 1,5-Bis (4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW 284c51, BW), physostigmine (Eserine), pyridostigmine, thapsigargin, actinomycin D and phorbol 12-myristate 13-acetate (PMA) were from Sigma Biochemicals (St. Louis, MI).

Cell cultures: Meg-01 cells were kindly provided by Dr. V. Deutsch (Tel Aviv, Israel) and cultured in Iscove's Minimal Dulbecco's Medium (IMDM) (Gibco-BRL), containing 10% heat-inactivated donor horse serum (DHS) in a fully humidified atmosphere at 37 °C in 5% CO_2. Half the media was replaced every 3 days. Cells were plated in a density of 1×10^6 cells/ml in six-well Nunclon (Nalge Nunc International, Denmark) plates, incubated with the noted agents for 18 h, fixed with fresh 4% paraformadehyde in phosphate-buffered saline (PBS, phosphate buffer 0.1 M; pH 7.4 and 0.9% NaCl) for 1 h, washed, resuspended in PBS and kept at 4 °C. Drugs (thapsigargin 10 nM, BIM 10 μM, H89 10 μM, physostigmine10 μM, ARP 3 nM) were administered alone or simultaneously with oligonucleotides. For immunocytochemistry or in situ hybridization, 25 μl samples of cell suspension were applied to 18 mm coverslips coated with poly-L-ornithine and allowed to dry at room temperature.

The RAW 264.7 macrophage cell line transformed with Abelson murine leukemia virus was obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated donor horse serum in a fully humidified atmosphere at 37 °C in 5% CO_2.

Oligonucleotides: EN101, a 20-mer anti-AChE mRNA antisense oligonucleotide ((AS-ON); 5′-ctgccacgttctcctgc-acc-3′), is targeted to exon 2 of human AChE mRNA, common to the AChE-S and AChE-R splice variants [23]. EN101 preferably induces destruction of nascent AChE mRNA transcripts [24]. EN101 was added directly to the cell medium at the final concentration of 3 nM. AChmiON, a fully 2′-*O*-methylated 23-mer oligonucleotide with the evolutionarily conserved sequence of human/murine miRNA-181a (5′-aacauucaacgcugucggugagu-3′ [25]) was from Microsynth GMBH (Balgach, Switzerland). AChmiON was added to the medium of Meg-01 cells to the final concentration of 0.1 μM, and cells were maintained in normal culture conditions for 24 h. Anti-miR-181, a 23-mer fully 2-*O*-methyl-protected AS ON (5′-acucaccgacagcguugaauguu-3′) served as a negative control to AChmiON.

RNA extraction, RT and real-time quantitative PCR: RNA was extracted from Meg-01 cells using the RNeasy kit (Beit Haemek, Israel) as per manufacturer's instructions. RNA concentration was verified by spectrophotometry. RT was performed using the Promega (Madison, WI) RT kit and 3′ primers for the human miR-181a precursor, human AChE-R and human actin. Quantitative real-time PCR [26] was